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Indian Standard

PHOSPHATE SOLUBILIZING FUNGAL INOCULANTS — *ASPERGILLUS AWAMORI* SPECIFICATION

ICS 65.080

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BUREAU OF INDIAN STANDARDS
MANAK BHAVAN, 9 BAHADUR SHAH ZAFAR MARG
NEW DELHI 110002

January 2009 Price Group 2

Soil Quality and Fertilizer Sectional Committee, FAD 7

FOREWORD

This Indian Standard was adopted by the Bureau of Indian Standards, after the draft finalized by the Soil Quality and Fertilizer Sectional Committee had been approved by the Food and Agriculture Division Council.

The phosphate solubilizing fungal inoculant *Aspergillus awamori* includes a culture, which efficiently converts the insoluble inorganic phosphate compounds into soluble form.

For the purpose of deciding whether a particular requirement of this standard is complied with, the final value, observed or calculated, expressing the result of a test or analysis, shall be rounded off in accordance with IS 2 : 1960 'Rules for rounding off numerical values *(revised)'*. The number of significant places retained in the rounded off value should be the same as that of the specified value in this standard.

AMENDMENT NO. 1 MAY 2012
TO
IS 15849 : 2009 PHOSPHATE SOLUBILIZING FUNGAL INOCULANTS — *ASPERGILLUS AWAMORI* — SPECIFICATION

[*Page* 1, *clause* **6.1**(j)] — Substitute ‘Net quantity in kg and area meant for;’ *for* ‘Net mass in kg and area meant for;’.

(FAD 7)

Reprography Unit, BIS, New Delhi, India

Indian Standard

PHOSPHATE SOLUBILIZING FUNGAL INOCULANTS — *ASPERGILLUS AWAMORI* — SPECIFICATION

1 SCOPE

This standard prescribes the requirements and methods of sampling and tests for *Aspergillus awamori* inoculants.

2 REFERENCES

The following standards contain provisions, which through reference in this text, constitute provisions of this standard. At the time of publication, the editions indicated were valid. **All** standards are subject to revision and parties to agreements based on this standard are encouraged to investigate the possibility of applying the most recent editions of the standards indicated below:

IS No.	*Title*
8268 : 2001	Rhizobium inoculants — Specification
14807 : 2000	Phosphate solubilizing bacterial inoculants — Specification

3 TERMINOLOGY

3.1 For the purpose of this standard the following definitions shall apply.

3.1.1 *Phosphate Solubilizing Fungal Inoculants — Aspergillus awamori (PSFI)* — PSFI is a product having a very high population (10^8-10^9 spores/g carrier) of *Aspergillus* sp. intended to solubilize inert/fixed form of phosphorus of the soil.

3.1.2 *Group of Crops* — PSFI is suitable for all types of crops as solubilizer of fixed phosphate in the soil.

4 REQUIREMENTS

4.1 When tested by the method prescribed in Annex A, PSFI shall contain 10^8 viable fungal spores/g of the carrier material on dry mass basis.

4.2 PSFI shall be carrier-based, colour depending on the colour of the carrier, carrier material, such as talc, carbox ymethyl cellulose (CMC), peat, vermiculite — soil-cow dung or compost may be used. It shall be neutralized *(pU* 7.0-7.5) with calcium carbonate and then sterilized. When tested it shall pass thorough 150 u sieve.

4.3 PSFI, when tested by the method prescribed in Annex A shall have no contamination with other micro organisms at 10^6 dilutions.

4.4 When tested by the method prescribed in Annex B of IS 8268, the pH of the PSFI shall be 6.5 to 7.5.

4.5 When tested by the method prescribed in Annex C of IS 14807, PSFI shall have good phosphate solubilizing capacity. Quantitatively solubilization capacity should be 30 percent minimum of the insoluble substrate (rock phosphate). The zone of solubilization should be at least 10 mm on Pikovskya medium of 3 mm thickness.

4.6 Specified mother culture to be obtained from any Institution maintaining the mother culture. The manufacturer is to control the quality of culture as given in Annex B.

4.7 Maintenance of Mother Culture

Mother culture may be maintained as slants of PDA having pure culture of *Aspergillus awamori* as inoculant. The slants of PDA inoculated with *Aspergillus awamori* are incubated at 30°C for 7 days so that mycelium and spores cover that entire agar surface. It is preserved under refrigerated conditions till further use. These cultures may be sub-cultured at regular intervals of 2-3 weeks for maintaining in active phase.

5 PACKING

PSFI shall be packed in polyethylene packs, thickness of which shall not be less than 100 u.

6 MARKING

6.1 Each polyethylene pack shall be marked legibly and indelibly with the following information:

a) Name of the product, specially as *Aspergillus awamori;*
b) Name and address of the manufacturer;
c) Crop(s) for which intended;
d) Type of the carrier used;
e) Batch number;
f) Date of manufacture;
g) Expiry date, which shall not be less than 6 months from the date of manufacture;
h) Strain(s) number;
j) Net mass in kg and area meant for;
k) Storage instructions worded as 'STORE IN A COOL PLACE AWAY FROM DIRECT SUNLIGHT AND HEAT'; and

m) Any other information required under the *Standards of Weights arid Measures (Packaged Commodities) Rules,* 1977.

6.2 Items (c), (f) and (g) above shall be printed oh a coloured ink background.

6.3 Direction for use of PSFI shall be printed briefly on the packets as given in Annex C. A separate pamphlet may preferably be given with it.

6.4 BIS Certification Marking

The product may also be marked with the Standard Mark.

6.4.1 The use of the Standard Mark is governed by the provisions of the *Bureau of Indian Standards Act,* 1986 and the Rules and Regulations made thereunder. The details of conditions under which the licence for the use of Standard Mark may be granted to manufacturers or producers may be obtained from the Bureau of Indian Standards.

6.5 Storage

PSFI shall be stored by the manufacturer in cool and dry place away from direct heat preferably at a temperature of 20°C and not exceeding 30°C It shall also be the duty of the manufacturer to instruct the retailers and, in turn, the users about the precautions to be taken during storage.

7 SAMPLING

The representative samples of the PSFI shall be drawn as prescribed in Annex F of IS 8268.

ANNEX A

(Clauses 4.1 *and* 4.3)

DETERMINATION OF NUMBER OF PHOSPHATE SOLUBILIZING FUNGAL SPORES/CFU

A-l APPARATUS

A-l.l Pipettes, Graduated, 1 ml and 10 mi.

A-1.2 Conical Flasks, 150 ml and 250 ml.

A-1.3 Screw-Capped Tubes, 10 ml.

A-1.4 Incubator

A-1.5 Petri Dishes

A-1.6 Hot Air Oven

A-1.7pH Meter

A-1.8 Autoclave

A-1.9 Haemocystometer

A-1.10 Compound Microscope

A-l.11 Glass Slides and Cover Slips

A-1.12 Forceps

A-1.13 Needles

A-1.14 Glass Rod

A-2 REAGENTS

A-2.1 Use a medium of die following composition:

Glucose	10.0 g
Tri-calcium phosphate	5.0 g
Ammonium sulphate	0.5 g
Magnesium sulphate	0.1 g
Sodium chloride	0.2 g
Yeast extract	0.5 g
Manganese sulphate	Trace
Ferrous sulphate	Trace
Distilled water	1000 ml
Agar	15.0 g
pH	7±0.2

A-2.2 Sterilizing and preparation process for plates are the same as described in Annex A of IS 8268.

A-3 PREPARATION OF SERIAL DILUTION FOR SPORE COUNT METHOD

Dispense 30 g of PSFI in 270 ml of sterile water and shake for 10 min on a reciprocal shaker. Make serial dilutions up to 10^{10} level. Pipette out 0.1 ml aliquots of 10^{6} to 10^{8} dilutions and deliver it on the petri dishes containing set medium as described in A-2.1. Spread the aliquots over the plate. Invert the plates and place them in die incubator at 28 ± 2°C for 3 days. Use 3 replicates of 10^{6},10^{7} and 10^{8} dilution.

A-4 COUNTING

Count the total number of colonies on the plates including colonies with solubilization zone with the help of colony counter.

A-5 METHOD FOR COUNTING SOLUBILIZATION ZONES

The process is the same as described in Annex C of IS 14807.

ANNEXE

(*Clause* 4.6)

GUIDELINES ON MAINTENANCE AND PREPARATION OF CULTURE AND QUALITY CONTROL

B-1 MAINTENANCE OF PURE CULTURES

Maintain pure culture of PSFI on the medium as described in A-2.1 in the form of slants. Alternatively cultures can also be maintained on Czapek's agar medium or on potato dextrose agar (PDA) prepared with the following constituents.

B.1.1 Czapek's Agar Medium

$NaN0_3$	3.0 g
$K,HP0_4$	1.0 g
$MgS0_4,7H_20$	0.5 g
KC1	0.5 g
$FeS0_4.7H_20$	0.01 g
Sucrose	30.0 g
Agar	15.0 g
Distilled water	1 litre

To reduce earamelization the sugar is added just prior to final sterilization. Sterilize at 15 psi at 121.6°C for 20 min.

B-1.2 Medium (PDA) for Mother Culture and Mass Multiplication

Potato (pelled and disced)	200 g/1
Glucose	20 g/1
Agar	15 g/1

Peeled potatoes are to be cut into small pieces. Boil 200 g of peeled and disced potatoes for 1 h in a litre of distilled water. Filter and make the volume to one litre. Add glucose and agar and steam until agar is dissolved.

NOTE — For both, agar is not added to the medium.

B-1.3 Mother Culture

It is generally supplied on PDA slants inoculated with pure culture of *Aspergillus awamori.*

B-1.4 Transfer a loopful of pure culture to each of the agar slants aseptically in an inoculation room and incubate at 28 ± 2°C for 3 days-. Always keep pure cultures below 5°C for storage.

B-2 PREPARATION OF INOCULUM CULTURE AND MASS CULTURE

B-2.1 Inoculum culture shall be prepared as described in **B-1** in Roux bottle slants using sterilized PDA medium.

B-2,2 Inoculation and Incubation

Inoculation of Roux bottle containing PDA medium is to be done by spore suspension of PSFI. The spore suspension is prepared in sterilized distilled water. Using a sterile glass rod gently, disturb the growth surface to remove the spores. Incubate the Roux bottles at 28-30°C temperature for 7-10 days. After 7-10 days of incubation, the entire surface of Roux bottle will have a lawn of spores.

NOTE - Incubation at 50° ± 1 °*C* speeds up the speculation process.

B-3 HARVESTING THE SPORES

The spores of the fungus should be harvested from fully-grown cultures in either petri plates or Roux bottles over PDA medium by adding sterilized distilled water or 0.1N sterile saline. Scrap the spores by gently disturbing the surface with a sterile glass rod. The spores suspension may be centrifuged at 5 000 rpm for 15 min and supernatant is to be discarded. The spore pellet is resuspended in a known amount of sterilized distilled water. To ensure uniform distribution of spores surfactant @ 0.2 percent (Tween 80 or Tween 20) is advised in the sterilized distilled water used. The spore count in the suspension is adjusted to 10^{12}/ml by the help of haemocystometer. Carboxy methyl cellulose can be added to the spore suspension and the volume adjusted such that it makes 2.0 percent solution and the spore suspension to 10^{6}/ml.

B-4 COUNTING

Count the total number of spores using haemocystometer using 10X magnification of the compound microscope.

B-5 QUALITY CONTROL TESTS RECOMMENDED AT BROTH/SOLID STAGE

B-5.1 Purity of *Aspergillus awamori* may be checked by the morphological characters. Colonies on Czapek's agar medium at 24-26°C, show diameter 405 cm in 10 days, deeply velvety.-plane, zonate, consisting of basal mycelium extending 2-3 mm beyond central area.

Conidial heads globose to radiate; 200-300 u (diameter); 1 u (wall thickness); vesicles globose. Colonies on malt extract agar medium at room temperature (24-26°C) grow rapidly reaching 6.0 cm in 10 days, heavily sporulating — brown spores showing a thin surface development of white mycelium, conidial heads are somewhat smaller and borne on considerably stouter stacks than on Czapek's medium but other dimensions similar to above described.

B-5.2 Qualitative Test

Check for freedom from visible contaminants by naked eyes or microscopically and observing solubilization zones. Morphological traits of the PSFI mentioned below may be used as criterion of purity.

ANNEX C

(*Clauses* 4.5 *and* 6.3)

DIRECTIONS FOR USE OF PHOSPHATE SOLUBILIZING FUNGAL INOCULANTS

C-l OPTIONS OF CARRIER MATERIALS

C-l.l Carrier Material: CMC, Talc, Calcium Carbonate

C-1,2 Soil: Charcoal (3:1)

C-1.3 Wheat Bran: Saw Dust: Tap Water (3:1:4, *v/v*)

This is to be taken in polypropylene bags and autoclaved for 1 h at 121.6°C for 3 successive days. The bags are inoculated with *Aspergillus awamori* and incubated at 30°C for 14 days. It is applied at the time of sowing as soil application (that is, to a depth of 7-10 cm).

C-1.4 Wheat Bran: Peat Formulation

Wheat bran:. Peat mixture (1:1 v/v) is to be autoclaved for 1 h. Moisture of the substrate to be adjusted to 50 percent *(w/w)* with sterile water medium inoculated with 0.1 ml of spore suspension of *Aspergillus awamori* containing 2 x 10^4 spores/ml and incubated for 7 days at 30°C. This can be used with rooting mixture for transplanting.

C-1.5 Vermiculite-Wheat Bran Formulation

Aspergillus awamori can be multiplied in PD broth for 10 days. Vermiculite (Grade-4) and milled wheat bran (250 mesh) are heated in hot air oven at 70°C for 3 days using metal pans, vermiculite 100 g, wheat bran (3.3 g), PD liquid broth culture (14 ml), and 0.05 N HO (17.5 ml) to be mixed and packed. This can be used for soil application.

C-2 MASS PRODUCTION TECHNOLOGY

500-ml conical flasks containing 150 ml of molasses-yeast medium or PD medium to be sterilized and inoculated with species of *Aspergillus awamori* and incubated for 10 days.

Approximately 10 litres of grown inoculum should be added to 200 litres of molasses-yeast medium in a fermenter and incubated for 10 days. Agitate the entire media to get broth culture mixed with talc powder (400 kg), air dried and sieved to get a fine powder and mixed thoroughly with cellulose (CMC) @ 5 percent packed in opaque polythene bags, heat sealed and store at room temperature. The minimum spore population should be 10^8 spores/ml. Final product should contain a minimum of 20 x 10^6 cfu/g.

NOTES

1 Sterilize the sampling and plating equipments with dry heat in a hot air oven at not less than 160°C for duration of not less than 2 h.

2 Sterilize the media by autoclaving at 121.6°C for 15 min. To allow the steam into and from closed containers when autoclaved, keep stoppers slightly loosened. Air from the chamber (autoclave) should be ejected allowing steam pressure to rise.

C-3 TALC BASED FORMULATIONS

Aspergillus awamori can be used in talc based carrier. To a well-blended 100 ml of PDA broth (inoculated with *Aspergillus awamori)* 250 g of sterilized talc power, 0.5 percent CMC and 3.5 g of calcium carbonate should be added. This can be thoroughly mixed and shade dried. Seeds are to be coated in talc based formulation @ 10 g/kg of seeds and shade dried for 2 h prior to showing.

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